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BIO-TECHNICAL METHODS SECTION (BTS)

BTS Leukemia

Frequent polymorphism in BCR exon b2 identified in BCR-ABL positive and negative individuals using fluorescent hybridization probes

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Recently, a polymorphic base in exon 13 of the BCR gene (exon b2 of the major breakpoint cluster region) has been identified in the eighth position before the junctional region of BCR-ABL cDNA. Cytosine replaces thymidine; the corresponding triplets are AAT (T allele) and AAC (C allele), respectively, both coding for asparagine. Therefore, this polymorphism has no implication in the primary structure of BCR and BCR-ABL proteins. However, since the alteration is located close to the fusion region it may have a significant influence on the annealing of PCR primers, probes for real time PCR, and antisense oligonucleotides. We have developed a RT-PCR-based screening method to easily identify polymorphic BCR and BCR-ABL alleles in CML patients and normal individuals in order to estimate their frequency. After amplification from cDNA, a melting curve of a specific fluorogenic probe mapping to the 3 end of BCR exon b2 and spanning the polymorphism readily discriminates between normal and polymorphic BCR and BCR-ABL alleles. This reporter probe is 3 labeled with fluorescein and placed next to 5 LC Red640-labeled anchor probes mapping to the 5 ends of BCR exon b3 or ABL exon a2 so that resonance energy transfer occurs when the probes are hybridized (LightCycler technology). T and C alleles were discriminated by a melting temperature difference of the reporter probe of 3.2 K. We have investigated cDNAs derived from leukocytes from seven cell lines and a total of 229 individuals: normal donors, *n* = 15; BCR-ABL negative chronic myeloproliferative disorders, *n* = 30; BCR-ABL negative acute leukemias, *n* = 11; b2a2$^{BCR-ABL}$ positive CML, *n* = 93; and b3a2$^{BCR-ABL}$ positive CML, *n* = 80. The frequency of the C allele was 33.0% in BCR-ABL negative individuals, 30.6% in b2a2$^{BCR-ABL}$, and 23.8% in b3a2$^{BCR-ABL}$ positive CML. In CML patients, 27.7% of BCR-ABL and 27.2% of BCR alleles had the C allele (NS). In total, 132 of 458 (28.8%) exons b2 of BCR or BCR-ABL alleles demonstrated this polymorphism. We conclude that a thymidine/cytosine replacement occurs frequently in BCR exon b2. Probes for real time quantitative RT-PCR should be designed not to map to the critical region in order to avoid underestimation of the number of BCR-ABL transcripts. *Leukemia* (2000) **14,** 2006–2010.
Keywords: chronic myelogenous leukemia; real time PCR; BCR gene; polymorphism analysis

Introduction

The two major players in the pathogenesis of CML, the proto-oncogene ABL on chromosome 9, and the breakpoint cluster region gene BCR on chromosome 22, have been identified, cloned and sequenced in the 1980s.[1,2] Various genomic polymorphisms have been described in context with the formation of the chimeric BCR-ABL fusion gene. However, the frequency of specific polymorphisms is still unknown.[2–5]

The BCR gene spans a region of 135 kb and comprises 23 exons, referred to as exons e1 to e23. Exons b1 to b5 of the major breakpoint cluster region (M-bcr) were identified as exons e12 to e16 of the BCR gene.[6,7] The breakpoint location within BCR falls either 5 between exons b2 and b3 or 3 between exons b3 and b4. A BCR-ABL fusion gene with a b2a2 or b3a2 junction is created and transcribed.[7]

Recently, a polymorphic base in exon 13 of the BCR gene (exon b2 of the major breakpoint cluster region) has been identified in the eighth position (Pos. 123692 GenBank accession No. U07000[2]) before the junctional region of BCR-ABL cDNA.[8] Cytosine replaces thymidine; the corresponding triplets are AAT and AAC, respectively, coding both for asparagine (Figure 1). Therefore, this polymorphism is silent and has no implication in the primary structure of BCR and BCR-ABL proteins. However, since the alteration is located close to the fusion region it may have a significant influence on the annealing of PCR primers, probes for real time PCR[5,9–13] and therapeutic antisense oligonucleotides.[14,15] Currently, the frequency of this polymorphism and therefore the potential impact is unknown.

To evaluate the frequency of this polymorphism and to analyze individual patients we established a novel screening method based on different melting temperatures of fluorescent-labeled oligonucleotide hybridization probes used for real time RT-PCR. Hybridization probes that bind to perfectly matched target sequences require a higher melting tempera-

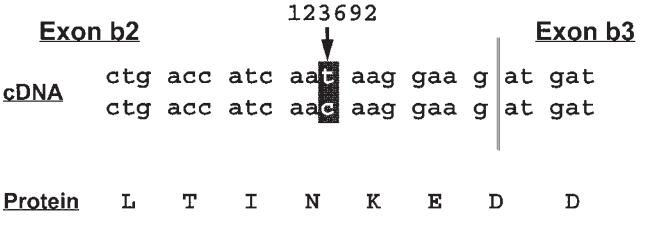

Figure 1 Polymorphic site in BCR exon b2 (T123962C) according to GenBank accession No. U07000. The single base change does not have any impact on the protein sequence of BCR and BCR-ABL proteins.

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ture (Tm) to dissociate compared to probes that bind to sequences with one or more mismatches.[16]

Materials and methods

Individuals and samples

A total of 229 individuals have been investigated for polymorphic BCR and/or BCR-ABL alleles. One hundred and seventy-three patients had BCR-ABL positive CML at diagnosis ($b2a2^{BCR-ABL}$, $n = 93$; $b3a2^{BCR-ABL}$, $n = 80$), 56 individuals were BCR-ABL negative (healthy donors, $n = 15$; BCR-ABL negative chronic myeloproliferative disorders, $n = 30$, BCR-ABL negative acute leukemias, $n = 11$). In addition, cell lines K562, LAMA84 ($b3a2^{BCR-ABL}$), BV173, KCL22, KYO1 ($b2a2^{BCR-ABL}$), HL60 and KG1a (BCR-ABL negative) were screened.

Sample processing, multiplex PCR for BCR-ABL transcripts

Total leukocyte RNA was extracted from 10 to 20 ml of peripheral blood after lysis of red blood cells or from 1 to 5×10^7 PBS washed cells from cell lines. RNA extraction was performed by CsCl gradient centrifugation or by commercially available extraction kits. RNA was reverse transcribed using random hexamer priming and MMLV reverse transcriptase as described.[17] The type of BCR-ABL transcript was determined by multiplex PCR[18] and electrophoresis of the PCR product on an ethidium bromide stained 1.5% agarose gel and compared with K562 ($b3a2^{BCR-ABL}$) and BV173 ($b2a2^{BCR-ABL}$) standards.

Mutation detection using fluorescence resonance energy transfer

Fluorescence monitoring using hybridization probes is based on the concept that a fluorescence signal is generated if fluorescence resonance energy transfer (FRET) occurs between two adjacent fluorophores.[9] Even more important, genotyping using two hybridization probes is possible with a shorter 'detection probe' recognizing an adjacent sequence. The greater stability of the longer 'anchor probe' causes the 'detection probe' to melt off the template at lower temperature so that polymorphic alleles can be distinguished by the melting temperature (Tm) of the 'detection probe'. Continuous fluorescence monitoring of the reaction as the temperature is raised from annealing to denaturation results in a sharp decrease in fluorescence at the temperature at which the 'detection probe' dissociates from the template. The single base change caused by the BCR polymorphism results in a decrease of Tm of the 'detection probe' that can be distinguished with the LightCycler.

Selection of primers and design of fluorogenic probes

The following primers were used for amplification: b2a2 and b3a2 BCR-ABL, primers B2A and NA4–;[9] BCR, primers B2A and c5e–.[18] The detection probe b2–17 (5 CTGACCAT CAATAAGGA-F) was a 17-mer oligonucleotide, mapping to the 3 end of BCR exon b2 and labeled at the 3 end with fluorescein. The 5 -LightCycler Red 640-labeled anchor probes were two 32-mer oligonucleotides that bind with a distance of three bases 3 to the detection probe. B3-32 maps to the 5 end of BCR exon b3 (5 LC Red640-TGATGAGTCTCCGGGGCTCTATGGGTTTCTGA-P), a2–32 to the 5 end of ABL exon a2 (5 LC Red640-AGCCCTT CAGCGGCCAGTAGCATCTGACTTTG-P). Therefore, detection of b3a2 BCR-ABL and BCR alleles was performed using probes b2–17 and b3–32, and detection of b2a2 BCR-ABL by using b2–17 and a2–32 (Figure 2).

Real time PCR and mutation detection

PCR was performed as described.[9] After amplification was complete, a final melting curve was recorded by cooling to 40°C with 10°C/s, holding at 40°C for 1 min and then heating slowly at 0.1°C/s until 65°C. The fluorescence signal (F) was measured continuously during the temperature rise to monitor the dissociation of the fluorescein-labeled detection probe and plotted against temperature (T) to produce melting curves for each sample (F *vs* T). Melting curves were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature against temperature (–dF/dT *vs* T). The actual Tm was calculated by the LightCycler software (version 3.0) (Figure 3). Amplification, fluorescence detection, and post-processing calculations were performed using the LightCycler (Roche Diagnostics, Mannheim, Germany).

Sequencing of amplification products

Eighteen randomly chosen amplification products of six BCR, six b2a2, and six b3a2 BCR-ABL alleles were sequenced (MWG Biotech, Ebersberg, Germany).

Quantification of BCR-ABL transcripts

In 20 $b2a2^{BCR-ABL}$ positive samples (10 each with the T and C allele) BCR-ABL transcripts were quantified using b2–17 and b3–32 probes and, for comparison, using a pair of hybridization probes mapping to ABL exon 3 as described.[9]

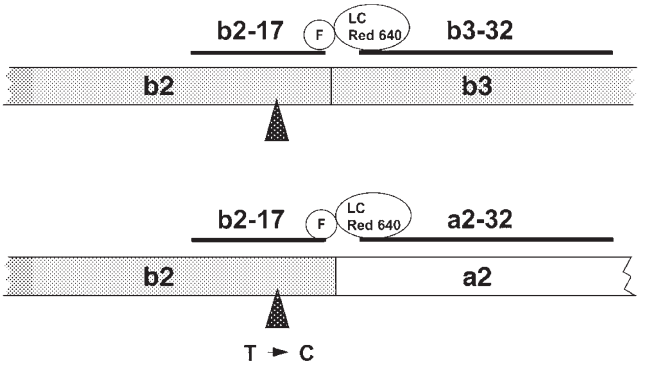

Figure 2 Schematic map of the primer probe combinations for the differential detection of BCR and BCR-ABL transcripts. The pair of hybridization probes map to exons b2 and b3 for the detection of b3a2 BCR-ABL and BCR alleles and to exons b2 and a2 for the detection of b2a2 BCR-ABL alleles, respectively. The unique detection probe b2–17 was 3 fluorescein labeled, the two anchor probes b3–32 and a2–32 were 5 LC Red 640 labeled.

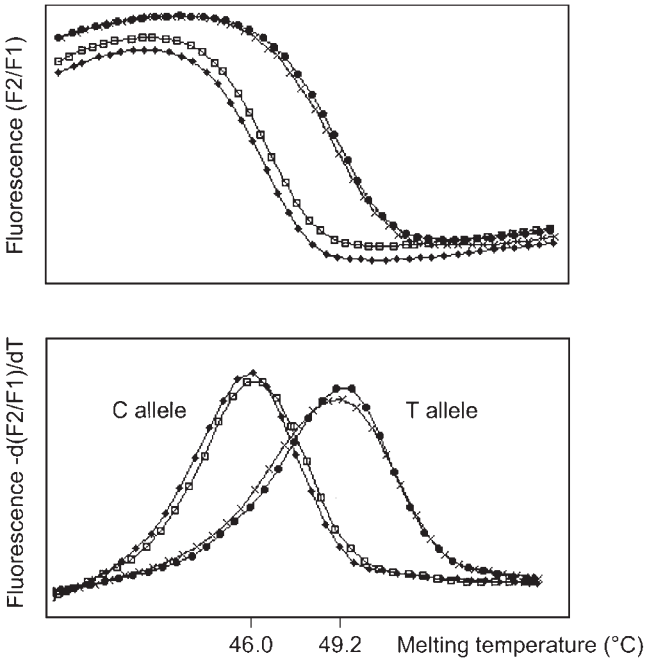

Figure 3 Melting curves [fluorescence (F2/F1) against temperature] and melting peaks [Fluorescence – d(F2/F1)/dT against temperature] of C and T BCR alleles. In four patients with BCR-ABL positive CML BCR alleles were amplified using primers B2A and c5e– and detected using the detection probe b2–17 and anchor probe b3–32. The Tm of the individual detection probes was 49.2°C in two cases (T alleles) and 46.0°C in two other cases (C alleles).

Statistical analysis

The statistical significance of the difference of the frequency of polymorphism between groups was calculated using Fisher's exact test (two-sided). The results of the BCR-ABL quantification (ratio between b2/b3 and a3/a3 labeling) were compared between samples with T and C alleles, respectively, using the Mann–Whitney test. Crude odds ratios were calculated and are given within the 95% confidence intervals. Whether or not the distribution of genotypes were in Hardy-Weinberg equilibrium was determined by ^{2} analysis.

Results

Melting temperature differences

The melting curves for BCR-ABL and ABL were identical since they were detected by the same probe (b2–17). Taking Mg^{2+}, oligonucleotide concentrations and the sequence for perfect match and mismatch into account a difference in the melting temperature between wild type and polymorphic allele of = 3.9 K was calculated according to Schütz and von Ahsen.[19]

Cell lines

Seven hematopoetic cell lines were investigated for polymorphic (T or C) BCR or BCR-ABL alleles. For the five BCR-ABL positive cell lines (BV173, KYO1, KCL22 – b2a2$^{BCR-ABL}$; K562, LAMA84 – b3a2$^{BCR-ABL}$) all five BCR-ABL alleles and four of five of the normal BCR alleles were of T type. The only C type allele was the BCR allele of LAMA84. Two BCR-ABL negative cell lines (HL60 and KG1a) showed heterozygous configuration of the BCR alleles. In total, 3/9 BCR alleles and 0/5 BCR-ABL alleles were of C type.

Samples of BCR-ABL positive patients and BCR-ABL negative individuals

Ninety-three b2a2 and 80 b3a2 BCR-ABL positive CML patients were analyzed. Forty-eight of 173 BCR-ABL alleles (27.7%), 47/173 BCR alleles (27.2%), and 95 out of a total of 346 BCR or BCR-ABL alleles (27.5%) had the C variant (Table 1).

Fifty-six BCR-ABL negative individuals were screened. Twenty-two individuals were homozygous for the T allele, 31 were heterozygous, and three were homozygous for the C allele. Overall, 37/112 BCR alleles had the C variant (33.0%) (Table 1).

In all individuals investigated, the frequency of the C allele in BCR or BCR-ABL was 132/458 = 28.8% (Table 1). The odds ratio for the frequency of polymorphic alleles in patients' populations revealed a lower rate of the C allele in b3a2$^{BCR-ABL}$ positive patients as compared to BCR-ABL negative individuals (OR = 1.84, $P = 0.03$, Table 2).

The overall frequency of the C or T alleles was 132/458 (p) and 326/458 (q), respectively. The expected frequencies of homozygous C, heterozygous, and homozygous T individuals was $p^2*458 = 19.03$, $2pq*458 = 93.96$, and $q^2*458 = 116.01$, respectively. The actual frequencies observed were 20 (8.7%), 92 (40.2%), and 117 (51.1%) (^{2} test NS) concluding that the polymorphism is in Hardy–Weinberg equilibrium.

Sequencing

Sequencing of six BCR, six b2a2 BCR-ABL, and six b3a2 BCR-ABL amplification products revealed the predicted sequence (T *vs* C) in all cases.

Impact on BCR-ABL quantification by real-time PCR

BCR-ABL transcripts were quantified in two cohorts of b2a2$^{BCR-ABL}$ positive samples with the T allele ($n = 10$) and with the C allele ($n = 10$) using b2–17 and b3–32 probes and by the standard method using a pair of probes mapping to ABL exon 3. The median ratio between the result of b2/b3 hybridization and a3/a3 hybridization was 0.43 (range 0.30–0.51) in the T allele cohort and 0.21 (range 0.07–0.51) in the C allele cohort, respectively (Mann–Whitney test, $P = 0.029$).

Discussion

Traditional methods for the detection of polymorphisms rely on conventional PCR amplification and subsequent detection of restriction fragment polymorphisms. These approaches are labor-intensive and depend on an appropriate restriction site and complete restriction digestion, which cannot always be achieved. To overcome these problems, we developed a simple and rapid assay using the LightCycler technology.

The LightCycler technology has been employed for several applications to detect hereditary single nucleotide mutations on DNA level in patients suffering from hemochromatosis,[20,21]

Table 1 Analysis of BCR polymorphism in peripheral blood samples from BCR-ABL positive and negative individuals

	Individuals	*Alleles*	*Polymorphism*				*BCR-ABL alleles C*		*BCR alleles C*		*C alleles total*	
	n	*n*	*T/T*	*BCR-ABL C/BCR T*	*BCR-ABL T/BCR C*	*C/C*	*n*	*%*	*n*	*%*	*n*	*%*
b2a2 BCR-ABL positive	93	186	48	20	13	12	32	34.4	25	26.9	57	30.6
b3a2 BCR-ABL positive	80	160	47	11	17	5	16	20.0	22	27.5	38	23.8
BCR-ABL positive	173	346	95	31	30	17	48	27.7	47	27.2	95	27.5
				BCR C/BCR T								
BCR-ABL negative	56	112	22	31		3	–	–	37	33.0	37	33.0
Total	229	458	117	92		20	48	27.7	84	29.5	132	28.8

Table 2 Odds ratio (OR) for the frequency of polymorphic alleles in patients' populations

	C allele/total	*OR*	*(95% CI)*	*P*
BCR-ABL negative	37/112	1		
BCR-ABL positive, total	95/346	1.30	(0.82–2.06)	NS
BCR-ABL allele	48/173	1.29	(0.77–2.15)	NS
BCR allele	47/173	1.32	(0.79–2.22)	NS
b2a2 BCR-ABL positive, total	57/186	1.12	(0.68–1.85)	NS
BCR-ABL allele	32/93	0.94	(0.53–1.68)	NS
BCR allele	25/93	1.34	(0.73–2.46)	NS
b3a2 BCR-ABL positive, total	38/160	1.84	(1.08–3.14)	0.03
BCR-ABL allele	16/80	1.91	(0.97–3.76)	NS
BCR allele	22/80	1.30	(0.69–2.44)	NS

1-antitrypsin deficiency,[22] hemoglobinopathies,[23] prothrombotic mutations (factor V Leiden,[20,24] prothrombin[24,25]), methylentetrahydrofolate reductase,[24,25] and inherited metabolic disorders.[26]

Here, we sought to establish a simple RT-PCR-based method to detect a BCR polymorphism that is also present in BCR-ABL. The unambiguous genotyping of polymorphic alleles has been performed by employing combinations of three primers and three hybridization probes.

With a frequency of the C allele of 29.7% in a Caucasian population, this polymorphism should be taken into account when choosing PCR primers, probes for real time PCR and antisense oligonucleotides. For quantitative PCR analysis, for example, probes spanning the b2/a2 fusion region might result in an underestimation of the number of BCR-ABL transcripts. Indeed, Branford *et al*[13] found that the apparent level of BCR-ABL copies as determined by a TaqMan assay was one order of magnitude lower in cases with the C allele in BCR-ABL. We confirmed the underestimation of BCR-ABL transcripts in C allele patients by comparing two real time PCR approaches: (1) using a pair of hybridization probes spanning the polymorphism and (2) mapping to ABL exon 3, respectively. Method (1) resulted in significant lower transcript numbers in C allele samples. Therefore, probes spanning this region should be avoided.[12,27]

In the past decade, antisense oligonucleotides[15,28,29] and ribozymes[30] have received attention for inhibiting BCR-ABL expression and colony formation derived from both human primary CML cells and BCR-ABL positive cell lines. The DNA–RNA heteroduplex formed by hybridization with antisense DNA is the substrate of ribonuclease H. However, sequence specificity is crucial for hybridization efficiency.[14] Therefore, antisense molecules spanning the b2/a2 fusion region and the polymorphic site[28,31,32] might be of limited value in a significant proportion of patients. All b2a2$^{BCR-ABL}$ cell lines that have been treated with antisense or ribozyme reagents have had the T allele in BCR-ABL and thus the influence of the polymorphism in this context is unknown.[33]

Investigating a higher number of individuals, we found a lower frequency of C alleles as compared to de V Meissner[8] (28.8% *vs* 43.5%). The significance of the lower proportion of C BCR-ABL alleles in b3a2$^{BCR-ABL}$ positive CML patients as compared to the frequency of C BCR alleles in BCR-ABL negative individuals (Fisher's exact test $P = 0.03$) and of less C BCR-ABL alleles in b3a2$^{BCR-ABL}$ *vs* b2a2$^{BCR-ABL}$ alleles (Fisher's exact test $P = 0.04$) is unclear.

We conclude that a considerable proportion of BCR and BCR-ABL alleles harbor a C/T replacement in BCR exon b2 of unknown biological significance. However, the base exchange may compromise quantitative PCR approaches or therapeutic strategies using specific antisense oligonucleotides. This polymorphism can easily be detected by a novel real time RT-PCR assay using specific fluorescent hybridization probes and should be taken into account when designing primers, probes or antisense oligonucleotides to BCR or BCR-ABL.

Acknowledgements

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